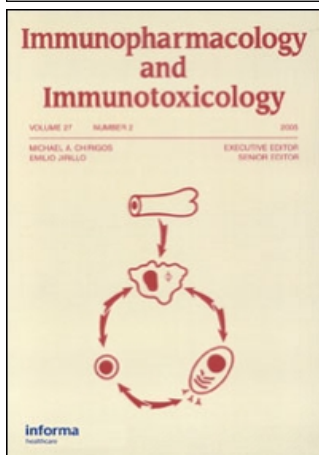


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## Immunopharmacology and Immunotoxicology

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### Effect of Skin Sensitizers on Inducible Nitric Oxide Synthase Expression and Nitric Oxide Production in Skin Dendritic Cells: Role of Different Immunosuppressive Drugs

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# Effect of Skin Sensitizers on Inducible Nitric Oxide Synthase Expression and Nitric Oxide Production in Skin Dendritic Cells: Role of Different Immunosuppressive Drugs

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Nitric oxide (NO) is involved in the pathogenesis of acute and chronic inflammatory conditions, namely in allergic contact dermatitis (ACD). However, the mechanism by which NO acts in ACD remains elusive. The present study focuses on the effects of different contact sensitizers (2,4-dinitrofluorbenzene, 1,4-phenylenediamine, nickel sulfate), the inactive analogue of DNFB, 2,4-dichloronitrobenzene, and two irritants (sodium dodecyl sulphate and benzalkonium chloride) on the expression of the inducible isoform of nitric oxide synthase (iNOS) and NO production in skin dendritic cells. It was also studied the role of different immunosuppressive drugs on iNOS expression and NO production. Only nickel sulfate increased the expression of iNOS and NO production being these effects inhibited by dexamethasone. In contrast, cyclosporin A and sirolimus, two other immunosuppressive drugs tested, did not affect iNOS expression triggered by nickel.

**Keywords** Cyclosporin A, Dexamethasone, iNOS, Nickel Sulfate, Nitric Oxide, Sirolimus, Skin Dendritic Cell.

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## INTRODUCTION

Dendritic cells (DCs) are ubiquitously distributed migratory antigen presenting cells, derived from CD34+ bone marrow stem cells that have the unique capacity to prime naïve T cells. DCs also regulate various effectors cell-functions and, therefore, play central roles in modulating the immune response, namely in allergic contact dermatitis (ACD).<sup>(1–3)</sup>

Among all the contact sensitizers, nickel is the most frequent cause of ACD. This hapten increases the expression of DC surface markers essential for antigen presentation, such as the molecules of the major histocompatibility complex class II and costimulatory molecules<sup>(4–10)</sup> and increases chemokine production and chemokine receptor expression<sup>(11–14)</sup>, thereby favoring antigen presentation. In DC, contact sensitizers activate mitogen-activated protein kinases<sup>(15–21)</sup>. We previously have reported that nickel sulfate activates the transcription nuclear factor kappa B (NF- $\kappa$ B) and the transcription factor activator protein-1 (AP-1) and induces the expression of inducible isoform of nitric oxide synthase (iNOS) in mouse skin DC<sup>(22)</sup>. However, the effect of other skin contact sensitizers and irritants on iNOS expression and nitric oxide (NO) production was never addressed before.

NO, a highly reactive radical produced from the amino acid L-arginine by the enzyme iNOS, mediates various physiological functions in the skin, ranging from the regulation of cutaneous blood flow to melanogenesis<sup>(23, 24)</sup>, and modulates antigen presentation.<sup>(25)</sup> If produced in excess, NO combines with superoxide anion to form peroxynitrite that has been considered responsible for tissue injury in skin inflammatory processes, such as ACD<sup>(26–30)</sup>. Therefore, in this work we studied the effect of different skin sensitizers that induce ACD on NO production by skin dendritic cells.

Sirolimus, cyclosporine A, and dexamethasone are clinically relevant immunosuppressive drugs with distinct molecular mechanisms of action, exhibiting different immunoregulatory profiles. Although the effect of these drugs on dendritic cell differentiation, migration, and maturation has been reported previously<sup>(31)</sup>, their direct effects on iNOS expression and NO production triggered by contact sensitizers remain unknown.

The present study focuses on the effects of different contact sensitizers (2,4-dinitrofluorbenzene, 1,4-phenylenediamine, nickel sulfate), the inactive analogue of DNFB, 2,4-dichloronitrobenzene (DCNB), and of two irritants (sodium dodecyl sulphate [SDS] and benzalkonium chloride [BC]), on the expression and activity of iNOS in skin DC. Moreover, we also studied the role of different immunosuppressive drugs on iNOS expression and NO production. As an experimental model of a DC we used a fetal skin-derived dendritic cell line (FSDC), that has morphological, phenotypical, and functional characteristics of immature skin DC, the Langerhans cells<sup>(32)</sup>.

## MATERIAL AND METHODS

Nickel sulfate, dexamethasone, benzalkonium chloride, and cyclosporin A were from Sigma Chemical Co. (Madrid, Spain). Sodium dodecyl sulphate was from GE Healthcare (Carnaxide, Portugal) and sirolimus was from Calbiochem (San Diego, CA, USA). The rabbit polyclonal anti-iNOS was from Abcam (Cambridge, UK). The alkaline phosphatase linked antirabbit IgG (H + L) antibody and the ECF substrate were purchased from GE Healthcare. Alexa Fluor 488-conjugated goat antirabbit antibody was from Molecular Probes (Leiden, The Netherlands) and the mounting medium for fluorescence, Vectashield, was obtained from Vector Laboratories, (Burlingame, CA, USA). The fetal calf serum was from Biochrom KG (Berlin, Germany) and trypsin from Invitrogen (Paisley, UK). The proteases inhibitor cocktail was from Roche (Carnaxide, Portugal). DCNB, DNFB, and phenylenediamine (PPD) were from Aldrich Chemical Company (Madrid, Spain). All other reagents were from Sigma Chemical Co.

### Cell Culture and Chemicals

The fetal mouse skin dendritic cell line FSDC was kindly supplied by Dr. G. Girolomoni<sup>(32)</sup> and cultured in endotoxin free Iscove's medium supplemented with 10 % (v/v) fetal calf serum, 1 % (w/v) glutamine, 3.02 g/l sodium bicarbonate, 100 µg/ml streptomycin, and 100 U/ml penicillin. For Western blot analysis FSDC were plated at  $2 \times 10^6$  cells/well in 6-well culture plates, for 24 hr, prior to treatment with the contact sensitizers or with the contact sensitizer nickel sulfate in the presence of the different immunosuppressors. For nitrite and MTT measurements the cells were plated at  $0.2 \times 10^6$  cells/well in 48-well culture plates in the presence of NiSO<sub>4</sub> and the immunosuppressors for 48 hrs. SDS (50 µg/ml), BC (1 µg/ml), PPD (50 µg/ml), and NiSO<sub>4</sub> (50 µg/ml) were dissolved in saline, while DNFB (1 µg/ml) and DCNB (1 µg/ml) were first solubilized in dimethyl sulphoxide. Dimethyl sulphoxide concentration never exceeded 0.025% in the culture medium.

### MTT Assay

Assessment of MTT reduction by metabolically active cells was made by a colorimetric assay, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as previously reported<sup>(33)</sup>. The cells ( $0.2 \times 10^6$  cells/well), cultured in 48-well microplates, were incubated for 48 hr with culture medium alone (control), with NiSO<sub>4</sub> (50 µg/ml), or with the contact sensitizer in the presence of the different immunosuppressors. After removal of cell-free supernatants for the nitrite assay, 400 µl of culture medium and 40 µl of MTT solution (5 mg/ml in PBS) were added to each well. The microplates were further incubated at 37°C for 1 hr. Supernatants were then discarded and 300 µl of acidified isopropanol (0.04 N HCl in isopropanol) were added to the cultures

and mixed thoroughly to dissolve the dark blue crystals of formazan. Formazan quantification was performed using an automatic plate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

### Nitrite Measurement

The production of NO was measured by the accumulation of nitrite in the culture supernatants, using a colorimetric reaction with the Griess reagent<sup>(34)</sup>. Briefly, after FSDC stimulation with culture medium alone (control), or with NiSO<sub>4</sub> (50 µg/ml), or with the contact sensitizer in the presence of the different immunosuppressors, for 48 hr, the culture supernatants were collected and diluted with equal volumes of the Griess reagent (0.1% [w/v] N-(1-naphthyl) ethylenediamine dihydrochloride, 1 % [w/v] sulphanilamide:5 % [w/v] H<sub>3</sub>PO<sub>4</sub>), during 10 min. The absorbance at 550 nm was measured after 20-min incubation in an automated plate reader (SLT). Nitrite concentration was determined from a sodium nitrite standard curve.

### Western Blot Analysis

For immunodetection of iNOS, cells were treated with culture medium (control), with the contact sensitizers NiSO<sub>4</sub> (50 µg/ml), DNFB (1 µg/ml), and PPD (50 µg/ml), with the irritants SDS (50 µg/ml) and BC (1 µg/ml), and with the inactive chemical DCNB (1 µg/ml). In another set of experiments, cells were treated with the contact sensitizer NiSO<sub>4</sub> in the presence of the different immunosuppressors, and with the immunosuppressors alone (data not shown), for 24 hr. After treatment, cells were washed with PBS and total cell lysates were obtained after harvesting the cells in a sonication buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM sodium orthovanadate, 1mM dithiothreitol, and the protease inhibitor cocktail. The lysates were then incubated on ice for 30 min and sonicated on ice at low amplitude (four times for 4 sec at 20 µm peak to peak) to disrupt the cells. Protein concentration was determined using the bicinchoninic acid method. Protein samples were separated on a 12% (v/v) SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% (w/v) dry milk in Tris-buffered saline with 0.1% (v/v) Tween 20, for 1 hr.

The level of iNOS protein was detected using a rabbit polyclonal anti-mouse iNOS antibody (1:200) for 1 hr, followed by incubation 1 hr at room temperature, with antirabbit IgG antibodies coupled to alkaline phosphatase, at 1:20,000 dilution. The immune complexes were detected using the ECF system (GE Healthcare), and the membranes were then scanned with blue-excited fluorescence on the Storm 860 (Amersham Biosciences), according to the manufacturer's instructions. The signals were analyzed using an image analyzer. To demonstrate equivalent protein loading the membranes were stripped and reprobed with an antiactin antibody (1:7500).

## Immunofluorescence Microscopy

For immunofluorescence analysis, FSDC cells grown on Lab-Tek chamber slide with cover ( $0.2 \times 10^6$  cells/slide) were treated, for 24 hrs, with the contact sensitizers  $\text{NiSO}_4$  (50  $\mu\text{g/ml}$ ), DNFB (1  $\mu\text{g/ml}$ ), and PPD (50  $\mu\text{g/ml}$ ), with the irritants SDS (50  $\mu\text{g/ml}$ ) and BC (1  $\mu\text{g/ml}$ ), and with the inactive chemical DCNB (1  $\mu\text{g/ml}$ ). In another set of experiments, cells were treated with the contact sensitizer  $\text{NiSO}_4$  in the presence of the different immunosuppressors, namely sirolimus (1 nM), dexamethasone (1  $\mu\text{M}$ ), and cyclosporin A (0.83  $\mu\text{M}$ ). The cells were then washed with PBS and fixed and permeabilized with methanol/acetone (1:1), for 10 min. Nonspecific binding was blocked by incubating the cells with 0.2 % gelatine in PBS supplemented with 0.1 % Tween 20, for 45 min at room temperature. Cells were then incubated for 120 min at room temperature, with a rabbit polyclonal antibody directed against iNOS (20  $\mu\text{g/ml}$ ).

After rinsing with PBS the cells were incubated with Alexa Fluor 488 conjugated goat antirabbit antibody (1:500) in 0.5% BSA-PBS, for 45 min. The chamber slide was rinsed and mounted with the mounting medium for fluorescence, Vectashield. Images from cells labelled with anti-iNOS were acquired on a Zeiss Axiovert 200 fluorescence microscope. Control experiments consisted of processing the same preparations as described, except for omission of the primary antibody, and resulted in no specific staining.

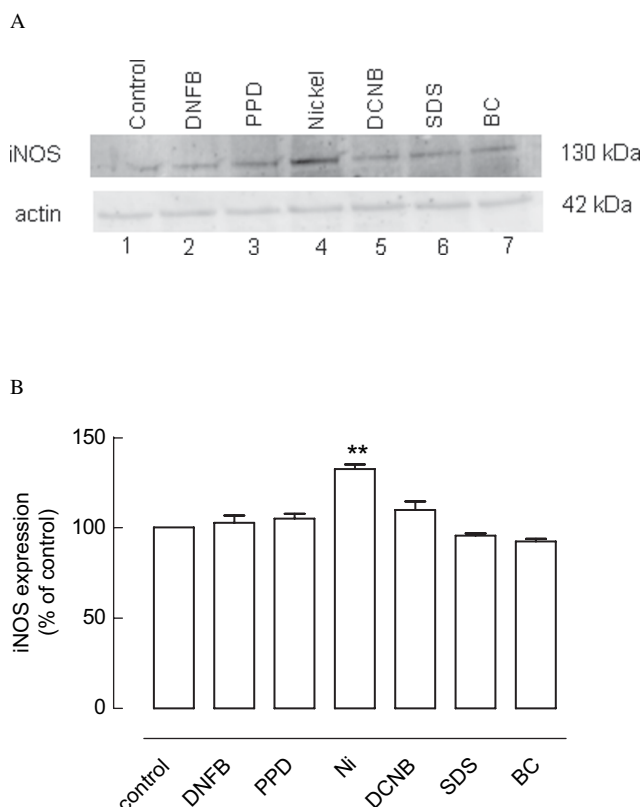
## Data Analysis

Results are presented as mean  $\pm$  SEM of the indicated number of experiments. Mean values were compared using one-way ANOVA followed by the Bonferroni's multiple comparison test. The significance level was 0.05.

## RESULTS

### Nickel Sulfate Increases iNOS Expression and NO Production

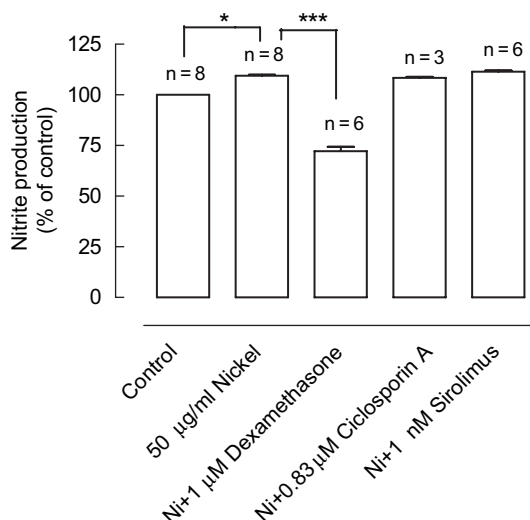
We evaluated the effect of the sensitizers nickel, DNFB, PPD, the irritants SDS, BC, and the nonsensitizer DCNB on the expression of iNOS protein in dendritic cells. Our results demonstrated that only nickel sulfate increased iNOS expression by FSDC (Figure 1, lane 4), detected by Western blot, and slightly increased nitrite production (Figure 2), determined by the Griess reagent. As shown in Figure 2, stimulation of the cells with  $\text{NiSO}_4$  for 48 hr increased nitrite production, from 100% when FSDC were incubated with culture medium alone, to  $109.5 \pm 1.2\%$ , when FSDC were incubated with 50  $\mu\text{g/ml}$   $\text{NiSO}_4$  ( $p < 0.05$ ), reflecting an increase in NO production. The assay of cellular MTT reduction did not show any significant toxic effect induced by  $\text{NiSO}_4$  for the concentration used in these experiments (data not shown).



**Figure 1:** Effect of contact sensitizers and irritants on iNOS expression in FSDC cells. FSDC cells ( $2 \times 10^6$  cells) were incubated in culture medium alone (control, lane 1), or with 1  $\mu$ g/ml DNFB (lane 2), 50  $\mu$ g/ml PPD (lane 3), 50  $\mu$ g/ml  $\text{NiSO}_4$  (lane 4), 1  $\mu$ g/ml DCNB (lane 5), 50  $\mu$ g/ml SDS (lane 6), or 1  $\mu$ g/ml BC (lane 7) for 24 hr. Total cell extracts were electrophoresed through SDS-PAGE, transferred to PVDF membranes and subjected to Western blot analysis using an anti-iNOS antibody. (A) The membranes were stripped and reprobed with an antiactin antibody to confirm equal protein loading (B). The results were quantified by scanning the membrane with a fluorescence scanner and analyzed using an image analyzer. Each value represents the mean  $\pm$  SEM from 4 experiments. \*\* $p < 0.01$ .

### Dexamethasone Inhibits iNOS expression and NO Production

We also investigated the effect of three immunosuppressive drugs, dexamethasone, cyclosporin A, and sirolimus, on iNOS expression and NO production stimulated by the contact sensitizer nickel sulfate on skin DC. After FSDC stimulation with the sensitizer nickel in the presence of dexamethasone (1  $\mu$ M), nitrite production induced by nickel was reduced to  $72.2 \pm 5.3\%$  of the control ( $p < 0.001$ ) (Figure 2). Cyclosporin A (0.83  $\mu$ M) and sirolimus (1 nM) did not modify the increase of nitrite production triggered by the contact sensitizer (Figure 2) ( $108.4 \pm 0.9$  and  $111.4 \pm 1.4\%$  of control, respectively). Western blot was used to examine the effect of immunosuppressive drugs on the



**Figure 2:** Effect of immunosuppressors on nitrite production induced by the contact sensitizer nickel sulfate in FSDC cells. FSDC cells ( $0.2 \times 10^6$  cells) were maintained in culture medium (control) or were incubated with 50 µg/ml  $\text{NiSO}_4$  or with  $\text{NiSO}_4$  in the presence of dexamethasone, cyclosporin A, or sirolimus, at the indicated concentrations, for 48 hr. Nitrite levels in the culture supernatants were detected by the Griess reaction as described in the Material and Methods section. Each value represents the mean  $\pm$  SEM from the indicated number of experiments, performed in duplicate. \* $p < 0.05$ , \*\*\* $p < 0.001$ .

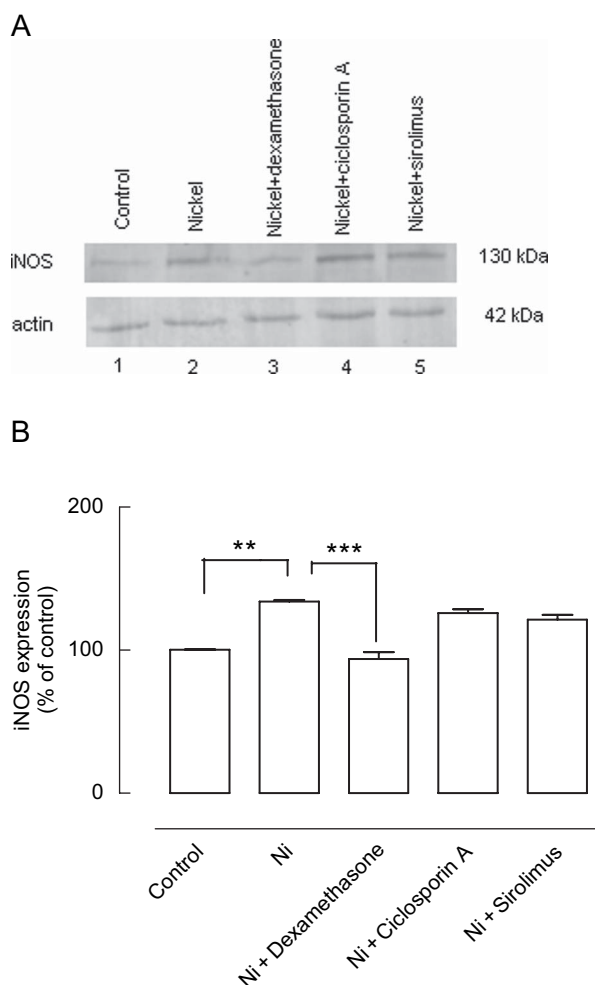
modulation of iNOS protein expression induced by  $\text{NiSO}_4$ . As indicated in Figure 3, nonstimulated cells (lane 1) expressed low levels of iNOS protein, but the expression of the protein increased after stimulation of cells with 50 µg/ml  $\text{NiSO}_4$  for 24 hr (lane 2). The basal expression of iNOS protein in control cells is probably due to the presence of fetal calf serum (10%) in the culture medium, which per se activates the cells to some extent.

Dexamethasone inhibited the expression of iNOS in cells stimulated with nickel sulfate (Figure 3, lane 3), as well as the basal iNOS expression. In contrast, neither cyclosporine A nor sirolimus modified the levels of iNOS protein (Figure 3, lanes 4 and 5, respectively). This decrease in protein expression caused by dexamethasone correlated well with the maximal inhibitory effect on nitrite production, as shown in Figure 2. The immunosuppressive drug concentrations used in this work were chosen based on the absence of toxicity to FSDC cells, as revealed by the MTT assay (data not shown). Dexamethasone slightly decreased the MTT reduction, to  $90.1 \pm 6.2\%$  ( $n = 3$ ) of the control, but this effect was not statistically significant (data not shown).

### Dexamethasone Inhibits iNOS Immunofluorescence

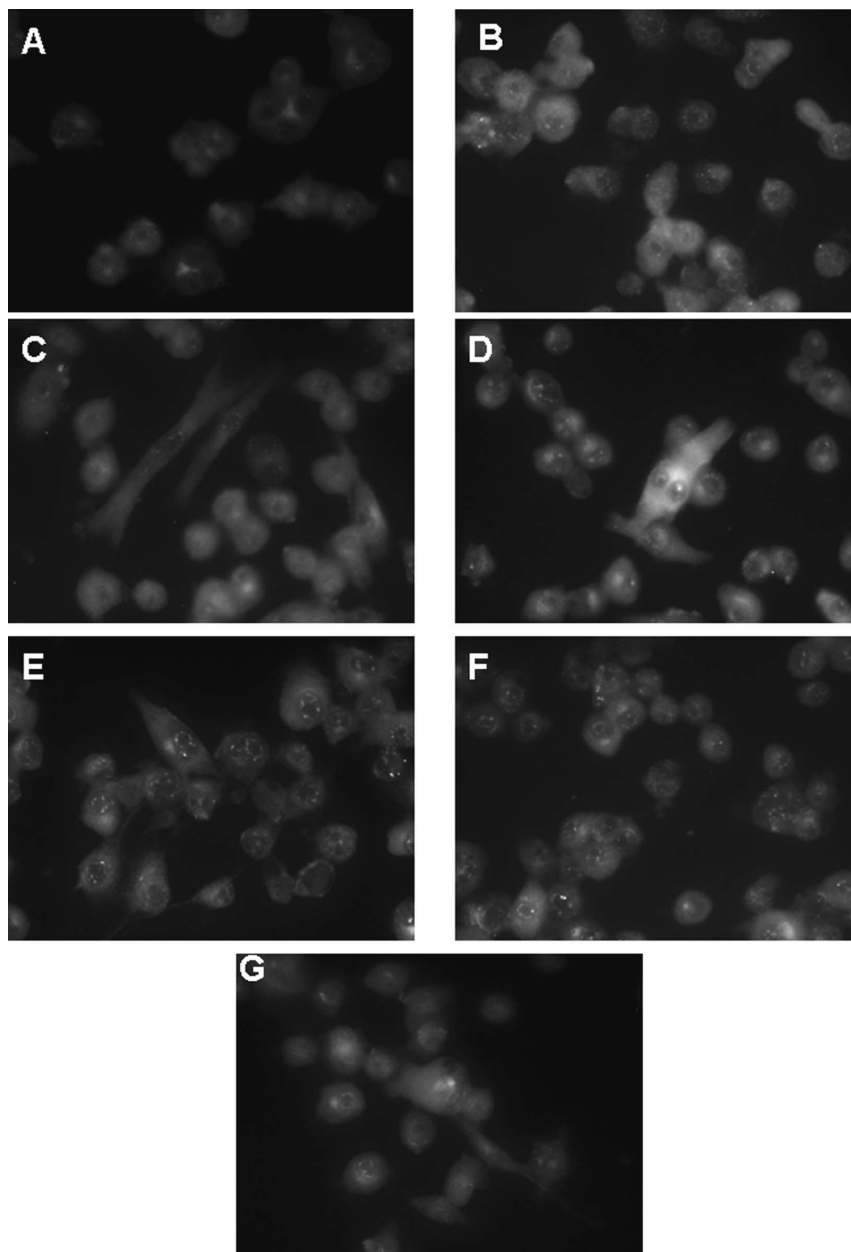
Immunofluorescent labelling of FSDC cells with the anti-iNOS polyclonal antibody was performed to confirm the results obtained by Western blot



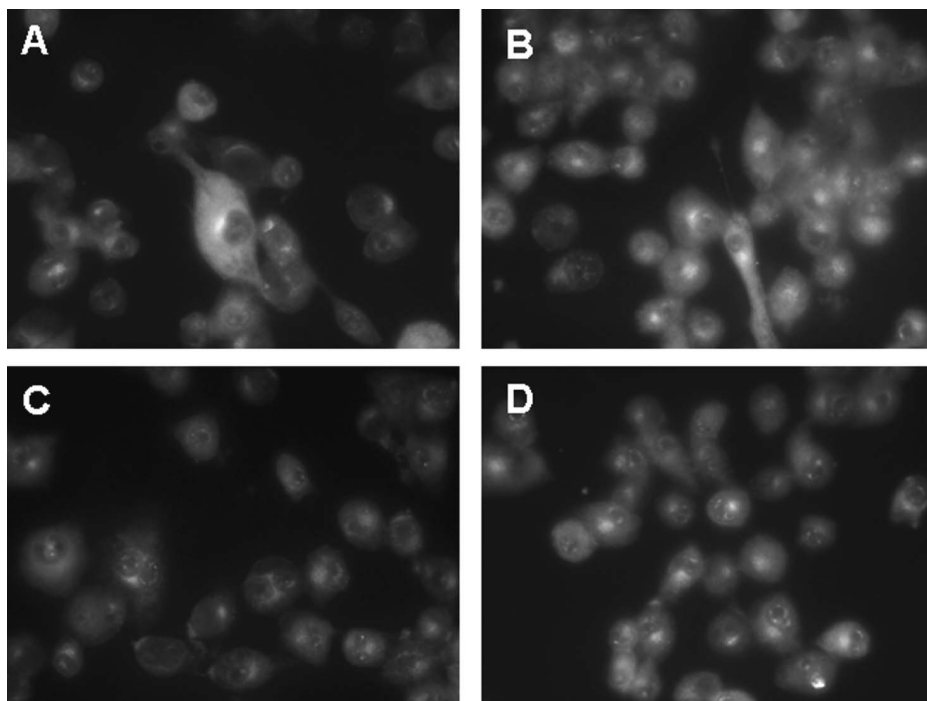


**Figure 3:** Effect of immunosuppressors on iNOS expression induced by the contact sensitizer nickel sulfate in FSDC cells. FSDC cells ( $2 \times 10^6$  cells) were incubated in culture medium alone (control, lane 1), with  $50 \mu\text{g/ml}$   $\text{NiSO}_4$  (lane 2), or with  $\text{NiSO}_4$  in the presence of  $1 \mu\text{M}$  dexamethasone (lane 3),  $0.83 \mu\text{M}$  cyclosporin A (lane 4), or  $1 \text{ nM}$  sirolimus (lane 5), for 24 hr. Total cell extracts were electrophoresed through SDS-PAGE, transferred to PVDF membranes, and subjected to Western blot analysis using an anti-iNOS antibody. (A) The membranes were stripped and reprobed with an antiactin antibody to confirm equal protein loading (B). The results were quantified by scanning the membrane with a fluorescence scanner and analyzed using an image analyzer. Each value represents the mean  $\pm$  SEM from 3 experiments. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

(Figure 4). Nickel sulfate slightly increased iNOS as compared with the cells maintained in culture medium. In addition, the other contact sensitizers and irritants studied, as well as DCNB, did not modify iNOS fluorescence. These results are in agreement with those obtained by Western blot (Figure 1) and indicate that the expression of iNOS is upregulated by the sensitizer  $\text{NiSO}_4$  in



**Figure 4:** Immunofluorescence of the iNOS protein in FSDC exposed to contact sensitizers and irritants. FSDC cells ( $0.2 \times 10^6$  cells) were maintained for 24 hr in culture medium, in the absence (control, A) or in the presence of 1  $\mu\text{g/ml}$  DNFB (B), 50  $\mu\text{g/ml}$  PPD (C), 50  $\mu\text{g/ml}$   $\text{NiSO}_4$  (D), 1  $\mu\text{g/ml}$  DCNB (E), 50  $\mu\text{g/ml}$  SDS (F), or with 1  $\mu\text{g/ml}$  BC (G). Immunostaining was performed as described in Materials and Methods section (magnification 650 $\times$ ).



**Figure 5:** Immunofluorescence of the iNOS protein in FSDC exposed to the contact sensitizer nickel and to immunosuppressors. FSDC cells ( $0.2 \times 10^6$  cells) were incubated, for 24 hr, with 50  $\mu\text{g/ml}$   $\text{NiSO}_4$  (A), with  $\text{NiSO}_4$  in the presence of 0.83  $\mu\text{M}$  cyclosporin A (B), 1  $\mu\text{M}$  dexamethasone (C), or 1 nM sirolimus (D). Immunostaining was performed as described in Materials and Methods section (magnification 650 $\times$ ).

FSDC. The results obtained by immunofluorescence also demonstrate that dexamethasone is the unique immunosuppressor able to decrease the iNOS expression triggered by the contact sensitizer nickel (Figure 5).

## DISCUSSION

The aim of our study was to evaluate the effect of the contact sensitizers DNFB, PPD, and nickel sulfate, and the irritants SDS and BC, on the iNOS protein expression to know if there is a different DC response that could discriminate between contact sensitizers and skin irritants. We have previously demonstrated, in a mouse skin dendritic cell line, that the contact sensitizer nickel sulfate increases iNOS expression and NO production in these skin dendritic cells<sup>(22)</sup>. However, the effect of other contact sensitizers and irritants on iNOS expression and NO production in DC was not addressed before. Our results demonstrated that only nickel sulfate increased iNOS expression by FSDC (Figure 1, lane 4, and Figure 4) and slightly increased nitrite production (Figure 2), reflecting an increase in NO production. Accordingly, other

researchers demonstrated that NiCl<sub>2</sub> and 2,4-dinitrochlorobenzene stimulate different signal transduction pathways in monocyte-derived dendritic cells and subsequently induce different phenotypic and functional changes in them<sup>(4)</sup>.

We also investigated the effect of three immunosuppressive drugs, dexamethasone, cyclosporin A and sirolimus, on iNOS expression and NO production induced by the contact sensitizer nickel sulphate, on skin DC. Dexamethasone was the only immunosuppressant that inhibited NO production (Figure 2) and iNOS expression (Figures 3 and 5D) triggered by nickel. Accordingly, dexamethasone, but not cyclosporin A, was previously shown to inhibit DC activation stimulated by different haptens, namely nickel<sup>(35)</sup>.

We have preliminary results ( $n = 2$ ) showing that nickel sulfate increases the expression of CD40 in this cell line and this increase was not inhibited by dexamethasone (1  $\mu$ M), cyclosporin A (0.83  $\mu$ M), or sirolimus (1 nM), which demonstrates that the inhibitory effect of dexamethasone on iNOS expression is specific to this protein.

The role of NO in numerous skin diseases, including wound healing, burn injury, ultraviolet light-induced sunburn erythema, psoriasis, irritant contact dermatitis and ACD has been extensively documented. However, the mechanism by which NO acts in ACD remains elusive<sup>(26, 36)</sup>. Hyun *et al.*<sup>(37)</sup> demonstrated that in a murine model of contact dermatitis, NO-releasing hydrocortisone NCX 1022 provided faster and greater protective effects than hydrocortisone alone. However, the researchers suggested that NO exerts a dual role in ACD, exerting both beneficial and aggravating effects<sup>(27)</sup>. Clinically, corticosteroids are among the first line drugs in the therapy of autoimmune and allergic skin diseases.

The present study highlights another mechanism, namely the inhibition of the NO production, which can be responsible for the anti-inflammatory properties of dexamethasone in skin dendritic cells. Accordingly, recent reports showed that pimecrolimus, a nonsteroid calcineurin inhibitor, has little effect on DC differentiation, maturation and immune function, in contrast to corticosteroids that showed pronounced effects on DC<sup>(38–41)</sup>. When compared with calcineurin inhibitors, namely cyclosporin A and tacrolimus, corticosteroids have a unique and profound inhibitory effect on the generation and function of DC<sup>(42)</sup>.

Manome *et al.*<sup>(43)</sup> demonstrated that dexamethasone and cyclosporin A affect differently the maturation of monocyte-derived DC but both reduced allostimulatory properties of DC despite induction of divergent molecular phenotypes<sup>(44)</sup>. Matsue *et al.*<sup>(45)</sup> also demonstrated contrasting effects of cyclosporin A, sirolimus, and dexamethasone on DC antigen presentation to T cells. These studies are in agreement with our results demonstrating that dexamethasone, sirolimus, and cyclosporin A affect differently NO production and iNOS expression induced by nickel sulfate in mouse skin dendritic cells

(Figures 2, 3 and 5). Dexamethasone was the only immunosuppressant able to inhibit NO production and iNOS expression.

The differential effect of immunosuppressant drugs on nickel-evoked up-regulation of NOS and nitrite production in FSDC is most likely related to their distinct mechanisms of action. Cyclosporin A binds to its intracellular immunophilin receptor (cyclophilin A) and this complex decreases calcineurin phosphatase-dependent nuclear translocation of the cytosolic nuclear factor of activated T cells<sup>(46)</sup>. Thus, the immunosuppressive effect of cyclosporin A is primarily due to its direct suppression of T-cell activation, although this drug may also have a direct effect on DC by mechanisms that are at least partly independent of calcineurin inactivation<sup>(31)</sup>. Our results showed that cyclosporin A did not modify the levels of iNOS protein (Figure 3, lane 4 and Figure 5C) and NO production (figure 2) elicited by the contact sensitizer nickel sulfate in FSDC. Accordingly, in rat hepatocytes, cyclosporin A also was without effect on iNOS expression and NO production triggered by interleukin 1 $\beta$ <sup>(47)</sup>.

Sirolimus (rapamycin) structurally resembles FK506 (tacrolimus). Although both bind to the same intracellular immunophilin, termed 12-kDa FK506-binding protein (FKBP12), the sirolimus-FKBP12 complex interacts with a distinct molecular target, known as mammalian target of rapamycin (mTOR), which results in the inhibition of multiple biochemical pathways critical for cytokine/growth factor-induced cellular proliferation, ribosome synthesis, translation initiation, and cell cycle progression into S phase<sup>(46)</sup>. Although sirolimus was shown recently to inhibit DC migration and DC-mediated T-cell activation<sup>(48)</sup>, our results indicated that sirolimus did not modify the levels of iNOS protein (Figure 3, lane 5, and Figure 5E) and NO production (Figure 2) elicited by nickel sulfate.

Glucocorticoids, on the other hand, exert their potent immunosuppressive and anti-inflammatory effects by inhibiting gene transcription, directly by competing for DNA-binding sites in the promoter regions, or indirectly by cross-coupling with many transcription factors, including the pro-inflammatory transcription factors AP-1 and NF- $\kappa$ B<sup>(49)</sup>. Since the promoter region of the iNOS gene contains binding sites for NF- $\kappa$ B and AP-1<sup>(50)</sup>, dexamethasone inhibits iNOS expression and NO production by interfering with the NF- $\kappa$ B and/or AP-1 signalling pathways<sup>(51,52)</sup>. Accordingly, our previous work demonstrated that, in FSDC cells, dexamethasone prevents granulocyte macrophage colony-stimulating factor (GM-CSF)-induced NF- $\kappa$ B activation, iNOS expression, and NO production<sup>(53)</sup>. The pathways regulating iNOS expression seem to vary in different cells or different species<sup>(50)</sup>.

We have previously demonstrated in this dendritic cell line (FSDC) and published elsewhere that iNOS expression induced by several stimuli, namely lipopolysaccharide, GM-CSF, and nickel sulphate, is clearly dependent on NF- $\kappa$ B activation<sup>(22,33,54,55)</sup>. Therefore, in this cell line this transcription factor is

critical for iNOS expression, whereas in other cells, namely macrophages, other transcription factors are also involved in iNOS expression, such as AP-1, Stat 1  $\alpha$ , IRF-1, CREB, and C/EBP.

Therefore, in this dendritic cell line iNOS expression is dependent on NF- $\kappa$ B activation and this justifies why only dexamethasone inhibited iNOS expression in contrast to cyclosporine and sirolimus whose principal molecular mechanism of action is to inhibit calcineurin phosphatase activity. Accordingly, nickel sulfate activates the NF- $\kappa$ B and AP-1 signalling pathways in FSDC<sup>(22)</sup>. NF- $\kappa$ B activation by nickel also was reported in human monocyte-derived DC<sup>(15)</sup> and in human endothelial cells<sup>(56, 57)</sup>. Moreover, in human airway epithelial cells, nickel-induced interleukin-8 expression was shown to be dependent on AP-1<sup>(58)</sup> and in epithelial cells nickel activates AP-1 through an oxidant-independent pathway<sup>(59)</sup>.

## CONCLUSION

We showed that nickel sulfate, in contrast with the effect of DNFB, PPD, and the irritants SDS and BC, increased iNOS expression in skin DC. Therefore, this endpoint cannot be used as an *in vitro* method to study the sensitization potential of chemicals or to discriminate between skin sensitizers and irritants.

Moreover, as the AP-1 and NF- $\kappa$ B transcription factors are involved in the iNOS expression induced by nickel sulfate, the inhibitory effect was observed only for dexamethasone and not for the other immunosuppressors studied, which do not significantly affect this pathway. This inhibitory effect of dexamethasone on the iNOS/NO pathway in DC, apart from a known effect on T cells and keratinocytes, may account for some of the anti-inflammatory properties of topical corticosteroids observed in inflammatory skin conditions<sup>(38–42)</sup>. A more pronounced inhibition of dexamethasone in the process of activation of DC by allergens, both during sensitization and the afferent phase of ACD elicitation, can sustain why medium/potent topical and systemic steroids are more effective in the treatment of ACD than the recently commercialized topical immunosuppressors, tacrolimus and pimecrolimus, which act through molecular mechanisms similar to cyclosporin A and sirolimus.

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